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SPECIAL ISSUE REVIEW PAPER

Regulation of cytokinin biosynthesis, compartmentalization and translocation

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Abstract

Cytokinins, a group of mobile phytohormones, play an important role in plant growth and development, and their activity is finely controlled by environmental factors in the control of morphogenic and metabolic adaptations. Inorganic nitrogen sources, such as nitrate, are a major factor regulating gene expression of adenosine phosphate-isopentenyltransferase (IPT), a key enzyme of cytokinin biosynthesis. Modulation of *IPT* and macronutrient transporter gene expression in response to nitrate, sulphate and phosphate, and cytokinin-dependent repression of the transporter genes suggest that cytokinins play a critical role in balancing acquisition and distribution of macronutrients. Biased distribution of *trans*-zeatin (tZ)-type cytokinins in xylem and N^6-(Δ^2-isopentenyl)adenine (iP)-type cytokinins in phloem saps suggest that, in addition to acting as local signals, cytokinins communicate acropetal and systemic long-distance signals, and that structural side chain variations mediate different biological messages. The compartmentalization of tZ- and iP-type cytokinins implies the involvement of a selective transport system. Recent studies have raised the possibility of subsets of the purine permease family as a transporter of cytokinin nucleobases and equilibrative nucleoside transporters (ENT) for cytokinin nucleosides. These biochemical and transgenic data suggest that AtENT6, an *Arabidopsis* ENT, could also participate in cytokinin nucleoside transport with a preference for iP riboside in vascular tissue.

Key words: Cytokinin, nitrogen, phloem, transporter, trans-zeatin, xylem.

Introduction

Cell division and differentiation take place in shoot and root meristems. Axes of cell division, tissue differentiation, and the subsequent elongation and expansion of cells ultimately determine the plant's shape. Regulation of these morphogenic events is influenced by environmental factors such as light, water, and nutrition. As a result, plant shape differs depending upon growth conditions, and individual plants form a unique shape within the genetic and developmental constraints of the species to take best advantage of its environment. To organize and maintain its body plan, plants must maintain the basic functions of the meristems and, at the same time, modulate their morphogenic and metabolic functions in response to environmental stimuli. Environmental conditions are transduced by a diverse array of signal molecules such as metabolites, phytohormones, peptide hormones, and RNA, some of which are transported through the plant via xylem vessels and sieve tubes.

Cytokinins are a group of mobile phytohormones that play a critical role in plant growth and development by regulating leaf senescence (Gan and Amasino, 1995; Kim *et al.*, 2006), apical dominance (Sachs and Thimann, 1967; Tanaka *et al.*, 2006), root proliferation (Werner *et al.*, 2001), phyllotaxis (Giulini *et al.*, 2004), reproductive competence (Ashikari *et al.*, 2005), and nutritional signalling (Takei *et al.*, 2001*b*, 2002). Recent studies on

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cytokinin metabolism and signal transduction have identified a series of genes involved in these processes and a model for the regulation of developmental processes by cytokinin-related genes has been proposed (Heyl and Schmülling, 2003; Kakimoto, 2003; Ferreira and Kieber, 2005; Sakakibara, 2006). It is also now known that cytokinins participate in the maintenance of meristem function (Werner *et al.*, 2003; Higuchi *et al.*, 2004; Leibfried *et al.*, 2005; Kurakawa *et al.*, 2007) and in the modulation of metabolism and morphogenesis in response to environmental stimuli (Sakakibara *et al.*, 2006; Werner *et al.*, 2006, see also references therein).

There is mounting evidence of complementary regulation between macronutrients and cytokinins for nutrient acquisition and distribution within the plant in response to environmental factors (Franco-Zorrilla *et al.*, 2002, 2004, 2005; Maruyama-Nakashita *et al.*, 2004*a*; Sakakibara *et al.*, 2006). Translocation of cytokinins is apparently mediated by subsets of purine permeases and nucleoside transporters by sharing the purine and the sugar conjugate transport systems, respectively (Gillissen *et al.*, 2000; Bürkle *et al.*, 2003; Hirose *et al.*, 2005). The question thus arises as to how a plant regulates cytokinin biosynthesis and distribution in response to internal and external environmental factors. In this article, recent discoveries are outlined about cytokinin metabolism and translocation, and the potential roles for cytokinins as local and long-distance signals are discussed.

Current model of cytokinin biosynthesis pathway

The naturally occurring cytokinins *trans*-zeatin (tZ), N^6-(Δ^2-isopentenyl)adenine (iP), *cis*-zeatin (cZ), and dihydrozeatin (DZ) are widely found in higher plant species (Mok and Mok, 2001; Fig. 1). These cytokinins differ in side chain structures, hydroxylation at the side chain terminus, and in the stereo-isomeric position and saturation of the isoprenoid side chain (Fig. 1). Among the four species, tZ and iP are most common in plants, but the physiological meaning of the differences in side chain structure are unclear.

The initial step of cytokinin biosynthesis is catalysed by adenosine phosphate-isopentenyltransferase (IPT). In higher plants, the major initial product is an iP nucleotide, such as iP riboside 5′-triphosphate (iPRTP) or iP riboside 5′-diphosphate (iPRDP) because the IPT predominantly uses dimethylallyl diphosphate (DMAPP) and ATP or ADP (Kakimoto, 2001; Sakakibara *et al.*, 2005). In *Arabidopsis*, iP nucleotides are converted into tZ

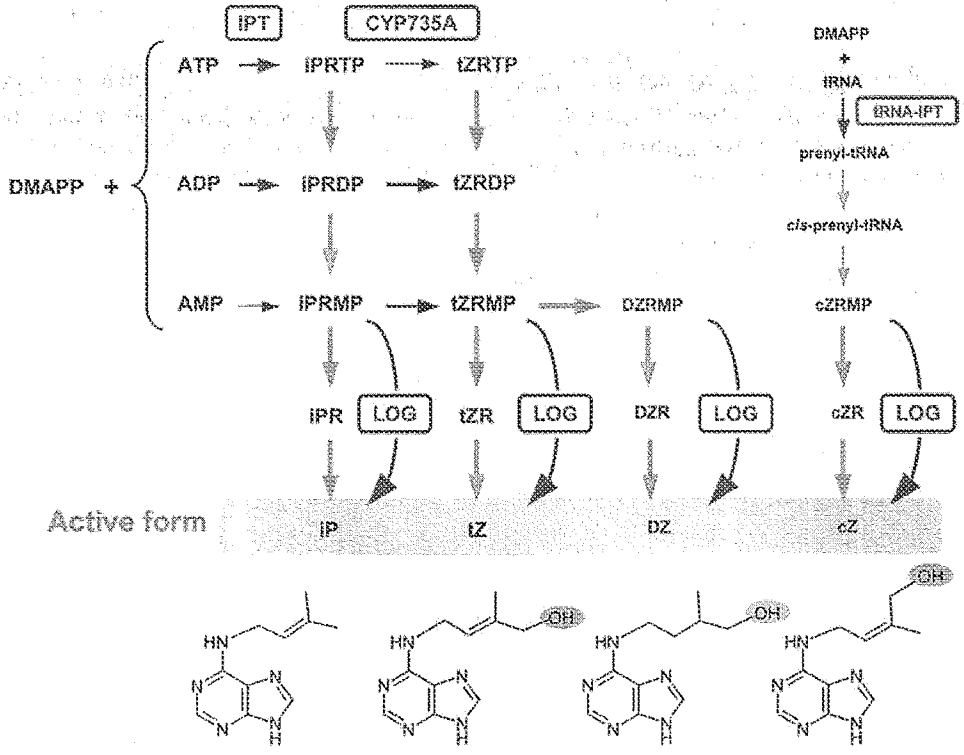


Fig. 1. A current model of cytokinin biosynthesis and the two known activation pathways. iPRMP, iP riboside 5′-monophosphate; tZRTP, tZ riboside 5′-triphosphate; tZRDP, tZ riboside 5′-diphosphate; tZRMP, tZ riboside 5′-monophosphate; DZRMP, DZ riboside 5′-monophosphate; cZRMP, cZ riboside 5′-monophosphate; DZR, DZ riboside; cZR, cZ riboside. Other abbreviations are as in the text. Blue arrows indicate reactions with known genes encoding the enzyme, and grey arrows indicate that the genes have not been identified. In this scheme, only biosynthesis and activation steps are drawn. Further details are shown in Sakakibara (2006).

nucleotides by cytochrome P450 mono-oxygenases CYP735A1 and CYP735A2 (Takei *et al.*, 2004*b*; Fig. 1). To become biologically active, iP- and tZ-nucleotides are converted to nucleobase forms by dephosphorylation and deribosylation, but genes encoding the nucleotidase (Chen and Kristopeit, 1981*a*) and nucleosidase (Chen and Kristopeit, 1981*b*) have not yet been identified. Recently, a novel pathway was identified that directly releases active cytokinin from the nucleotide, catalysed by the cytokinin nucleoside 5′-monophosphate phosphoribohydrolase called LOG (Kurakawa *et al.*, 2007; Fig. 1). Thus, it is likely that there are at least two cytokinin activation pathways in plants, although any functional differentiation between the pathways remains to be clarified.

Physiological differentiation of *IPTs*

IPT is encoded by a small multi-gene family in *Arabidopsis* (*AtIPT1*, *AtIPT3–AtIPT8*; Kakimoto, 2001; Takei *et al.*, 2001*a*) and rice (*OsIPT1–OsIPT8*; Sakamoto *et al.*, 2006). tRNA-isopentenyltransferase (tRNA-IPT) is primarily involved in maturation of tRNA species (Golovko *et al.*, 2002), and is also involved in cZ formation in *Arabidopsis* (Miyawaki *et al.*, 2006; Fig. 1). Analyses of the individual *IPT*s revealed that *AtIPT3*, which is predominantly expressed in the phloem, is up-regulated by nitrate (Miyawaki *et al.*, 2004; Takei *et al.*, 2004*a*). The nitrate-dependent accumulation of cytokinin was greatly reduced in an *ipt3* mutant, indicating that *AtIPT3* is responsible for nitrate-dependent cytokinin biosynthesis (Takei *et al.*, 2004*a*). An *AtIPT3 promoter–reporter* construct in transgenic *Arabidopsis* showed that the response is transcriptionally regulated (Fig. 2A; Miyawaki *et al.*, 2004). Other macronutrients, such as sulphate and phosphate also modulate the accumulation of *AtIPT3* transcripts (Fig. 2B). Genome-wide microarray assays indicate that cytokinin application significantly represses genes for subsets of macronutrients (i.e. nitrate, sulphate,

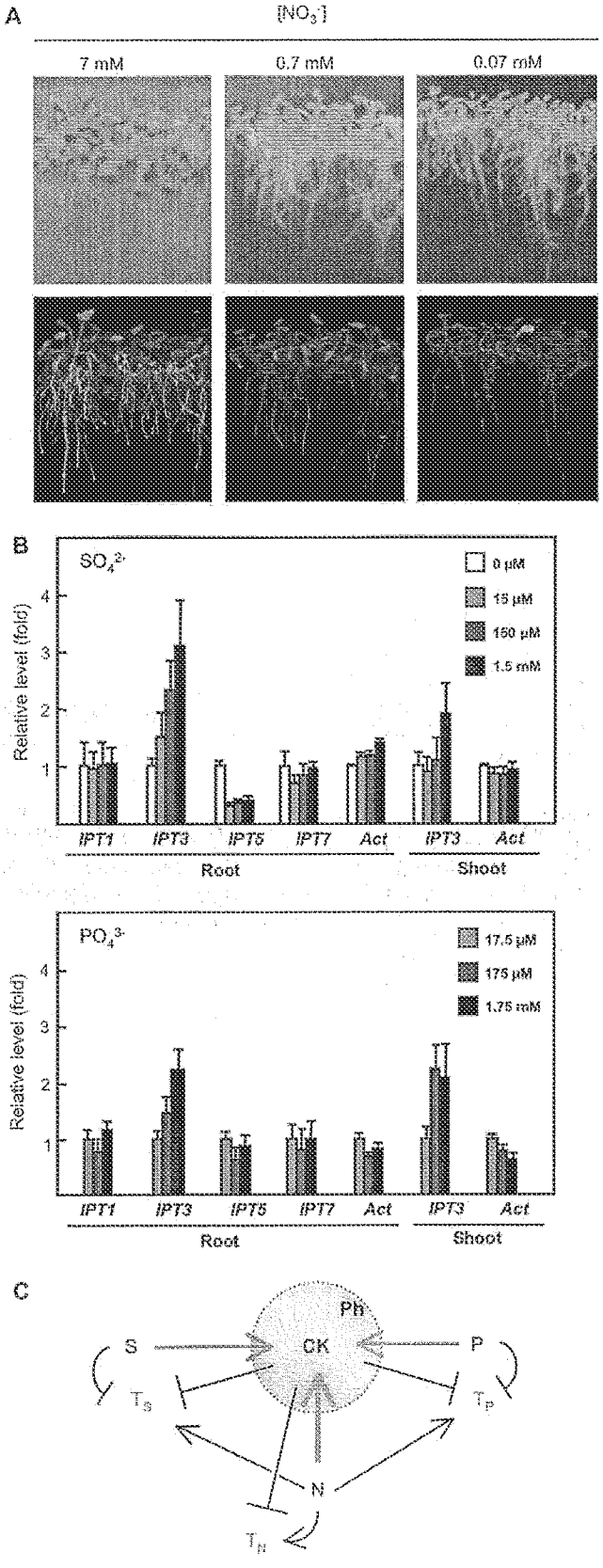


Fig. 2. Effect of macronutrients on *IPT* expression. (A) Nitrate concentration-dependent fluorescence in IPT3 promoter:GFP transgenic *Arabidopsis*. Transgenic *Arabidopsis* plants were grown on agar plates with MGRL salts (Fujiwara *et al.*, 1992) supplemented with 1% sucrose for approximately 2 weeks. Details of the transgenic line are described in Takei *et al.* (2004*a*). (B) Effects of growth medium sulphate and phosphate concentrations on the expression of *AtIPTs*. *Arabidopsis* seedlings were grown for 11 d after germination on MGRL medium at the indicated sulphate (upper panel) or phosphate (lower panel) concentrations. Transcript accumulation is given as relative values with one unit defined as the accumulation at the lowest concentration. Other experimental conditions are described in Takei *et al.* (2004*a*). (C) Schematic representation of the regulatory relationship among macronutrients, cytokinin biosynthesis and macronutrient transporters. N, nitrate; S, sulphate; P, phosphate; T_N, nitrate transporter gene; T_S, sulphate transporter gene; T_P, phosphate transporter gene; CK, cytokinin; Ph, phloem. Arrowhead bar indicates positive regulation, and flat bar indicates negative regulation.

and phosphate), transporters in *Arabidopsis* (Brenner *et al.*, 2005) and rice (Hirose *et al.*, 2007), and a subset of nitrate transporter genes is induced by nitrate, whereas a subset of sulphate and phosphate transporters are repressed by their substrates (Grossman and Takahashi, 2001; Wang *et al.*, 2003, 2004; Scheible *et al.*, 2004). For instance, expression of *NRT2;1*, which functions as a high affinity nitrate transporter and also as a key regulator of root system architecture, is induced by nitrate but repressed by cytokinin (Wang *et al.*, 2003, 2004; Scheible *et al.*, 2004; Brenner *et al.*, 2005). Expression of the sulphate transporters *SULTR1;1* and *SULTR1;2* and a phosphate transporter *PHT1;1* are up-regulated by sulphate and phosphate starvation, respectively, but are down-regulated by the application of cytokinin (Franco-Zorrilla *et al.*, 2002, 2004, 2005; Maruyama-Nakashita *et al.*, 2004*b*). Differential regulation of transporter genes by their substrates, up-regulation of *IPT* by macronutrients, and the repression of transporter genes by cytokinin imply that cytokinin plays a key role as a feedback signal to maintain acquisition balance among the macronutrients (Fig. 2C). In addition, the spatial expression patterns of transporter genes (i.e. *NRT2;1* and *SULTR1;2* in epidermal and cortical cells; Maruyama-Nakashita *et al.*, 2004*b*; Nazoa *et al.*, 2003) do not always overlap with *AtIPT3* (i.e. phloem; Miyawaki *et al.*, 2004; Takei *et al.*, 2004*a*). Thus, a cytokinin transport system could be involved in distant signal communication.

On the other hand, *AtIPT7* is positively controlled by a KNOX-type regulator, which functions to form and maintain the shoot apical meristem (SAM) in *Arabidopsis* (Jasinski *et al.*, 2005; Yanai *et al.*, 2005), and *OsIPT2* and *OsIPT3* in rice (Sakamoto *et al.*, 2006). The response of different sets of *IPT*s to nutrition status and transcriptional regulators suggests that *IPT*s are functionally specialized either to produce cytokinins in response to macronutrients to modulate metabolism and morphogenesis, or to supply a basic level of cytokinin to maintain SAM function. The nutrient-dependent regulation of *AtIPT3* in phloem would have an additive effect on the SAM to fine-tune hormone action for morphogenic adaptation.

Cytokinin as local and long-distance signals

The spatial expression patterns of promoter–reporter gene fusions in transgenic *Arabidopsis* lines harbouring cytokinin biosynthesis (i.e. *AtIPT*; Miyawaki *et al.*, 2004; Takei *et al.*, 2004*a*), degradation (*cytokinin oxidase*, *AtCKX*; Werner *et al.*, 2003, 2006), and signalling (*response regulator*, *ARR*; Mason *et al.*, 2004; Ferreira and Kieber, 2005) indicate that cytokinin can be locally synthesized, act as an autocrine or paracrine signal, and can be catabolized at distant sites (Fig. 3). A phenotypic response to conditional induction of *ipt* in tobacco supports the idea

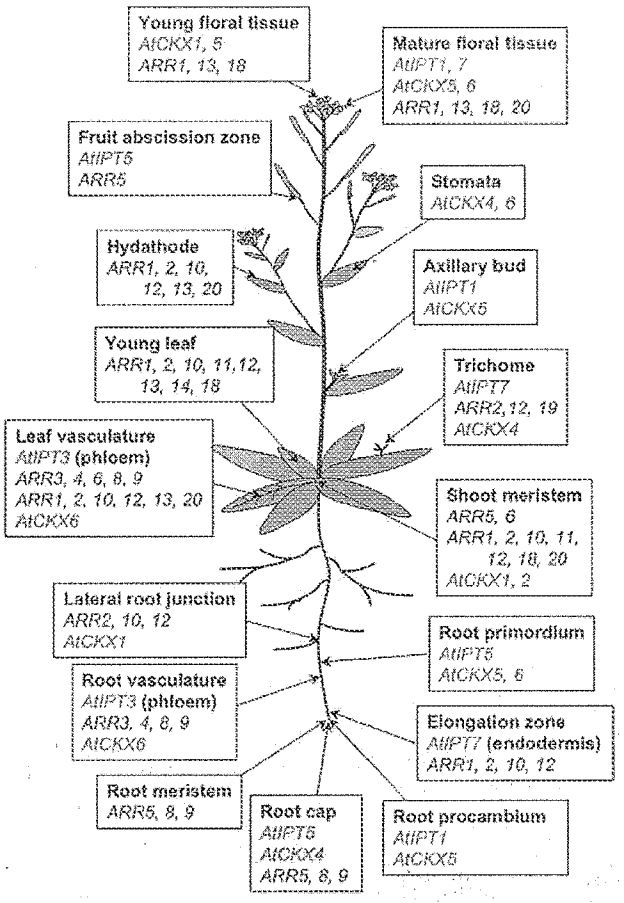


Fig. 3. Spatial distribution of cytokinin-related gene expression in *Arabidopsis*. Data are based on studies of *promoter:GUS* (Miyawaki *et al.*, 2004) and *promoter:GFP* (Takei *et al.*, 2004*a*) fusions of *AtIPT*s (red), Type A *ARR*s (violet; D'Agostino *et al.*, 2000; Kiba *et al.*, 2002, 2003; Ferreira and Kieber, 2005; Yokoyama *et al.*, 2007), Type B *ARR*s (blue; Mason *et al.*, 2004; Tajima *et al.*, 2004), and *AtCKX*s (green; Werner *et al.*, 2003, 2006).

that local induction of *Agrobacterium IPT* expression in lateral buds causes the growth of single buds, but only at the site of induction (Faiss *et al.*, 1997).

Cytokinins have also been thought to act as a long-distance signal because it was found in the xylem sap of several different plants (Yong *et al.*, 2000; Morris *et al.*, 2001; Takei *et al.*, 2001*b*; Kuroha *et al.*, 2002; Kudoyarova *et al.*, 2007). Thus far, the major forms of cytokinin in xylem sap are the tZ-type cytokinins, such as tZ riboside (tZR). Expression analysis of *CYP735A1* and *CYP735A2* suggests that *CYP735A2*, which is the major one, is predominantly expressed in roots, and only faint expression is observed in aerial parts (Takei *et al.*, 2004*b*). These results suggest that roots are a major site of tZ production, and that tZR plays a role as a root-to-shoot acropetal signal. These measurements of cytokinin in xylem and phloem sap of *Arabidopsis* support this idea (Table 1). Xylem sap predominantly contains tZ-type cytokinins,

Table 1. *Biased distribution of iP and tZ-type cytokinins in the xylem and phloem of Arabidopsis*

Xylem and phloem sap were collected from *Arabidopsis* plants grown on Rockfiber blocks (Nittobo, Tokyo) by a micropipetting (Pilot *et al.*, 2004) and an EDTA method (Lejeune *et al.*, 1994), respectively. Data are from two independent experiments (Exp. 1 and Exp. 2). Cytokinins and other compounds were measured by the method of (Yonekura-Sakakibara *et al.*, 2004) and (Soga and Imaizumi, 2001), respectively. n.d., not detected. Other abbreviations are as in the text.

Compound	Xylem sap (xylem exudate)		Phloem sap (leaf exudate)	
	Exp. 1 (fmol plant^{-1} h^{-1})	Exp. 2 (fmol plant^{-1} h^{-1})	Exp. 1 (fmol leaf^{-1} h^{-1})	Exp. 2 (fmol leaf^{-1} h^{-1})
iP	2.9	5.2	0.1	0.5
iPR	5.3	15.1	15.2	9.4
tZ	4.1	30.9	n.d.	0.3
tZR	65.1	64.3	0.8	1.0
cZ	6.7	2.6	0.3	1.0
cZR	9.7	4.9	7.6	17.3
	(mM)	(mM)	(mM)	(mM)
Sucrose	–	n.d.	–	2.61
Glucose	–	0.12	–	0.88
Nitrate	–	21.5	–	n.d.

and phloem sap predominantly contains iP-type and cZ-type cytokinins. Biased distribution of iP-type cytokinins in phloem sap has also been reported (Corbesier *et al.*, 2003). Phloem cytokinins might function as a basipetal or as a systemic signal. Such compartmentalization might be needed for plant cells to recognize the direction of signal mediated by cytokinins.

Results from studies on grafted mutants imply that there is feedback regulation of xylem sap cytokinins (i.e. tZR) by some long-distance signal that moves from shoot to root (Beveridge *et al.*, 1997; Foo *et al.*, 2007). The *rms4* mutant in pea, which confers a phenotype with increased shoot branching, has lower concentrations of tZR in the xylem sap (Beveridge *et al.*, 1997). Grafting experiments showed that the regulatory signal comes from the shoot (Beveridge *et al.*, 1997). The homeostatic mechanisms that control xylem cytokinin levels appear to be common in *Arabidopsis* and pea (Foo *et al.*, 2007). Although the chemical nature of the shoot-to-root signalling substance is not clear, it will be essential to characterize the long-distance signalling system to understand the biological importance of xylem and phloem cytokinins.

Ligand preference of cytokinin receptors

At present, the biological significance of compartmentalization of tZ-type and iP-type cytokinins in the xylem and phloem is unclear. However, differences in the ligand preference of cytokinin receptors could yield some clues. There are at least three cytokinin receptors in *Arabidopsis*: AHK2, AHK3, and AHK4/CRE1/WOL (Mähönen *et al.*, 2000; Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Ueguchi *et al.*, 2001; Yamada *et al.*, 2001). In an assay for receptor–ligand affinity using an *Escherichia coli* expression system (Suzuki *et al.*, 2001; Romanov *et al.*, 2005), both AHK3 and AHK4/CRE1/WOL bound tZ with similarly high affinity, but AHK3 had much less affinity to iP than AHK4/CRE1/WOL (Spíchal *et al.*, 2004; Romanov *et al.*, 2006). It is noteworthy that *AHK3* is expressed predominantly in shoots and *AHK4/CRE1/WOL* is expressed predominantly in roots (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004). Such different properties could confer specialized functions on the receptors, and make sense of the role that side chain variations play in signalling.

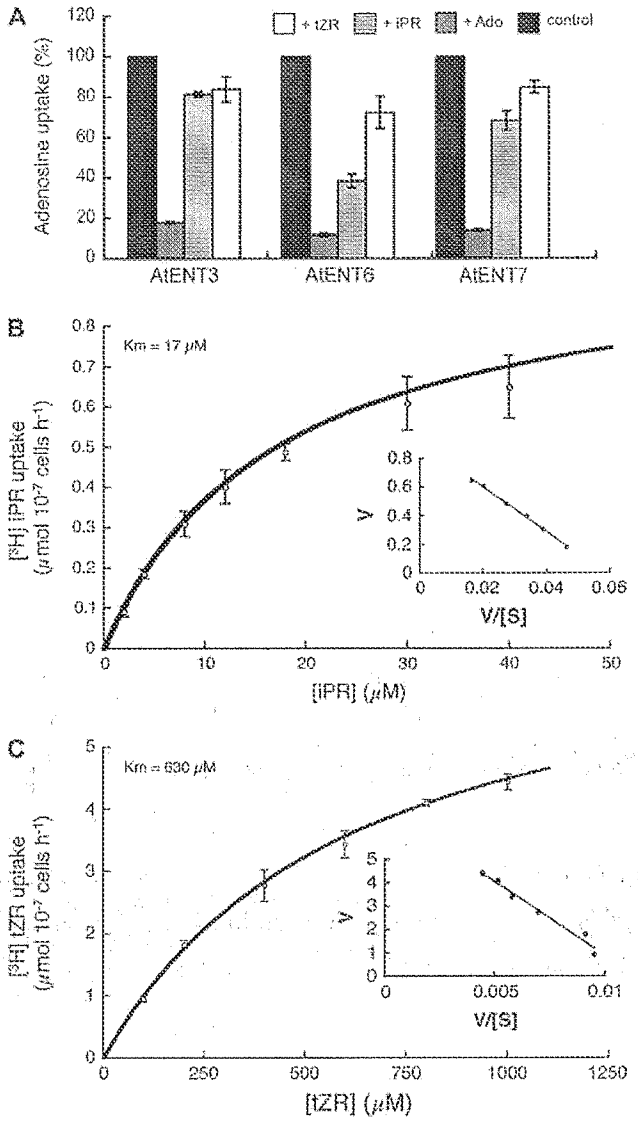
Candidates for cytokinin transporters

Biased distribution of tZ and iP between xylem and phloem implies that some system organizes the appropriate distribution of cytokinin species in the plant body. Although the main route of cytokinin biosynthesis is catalysed by enzymes that specifically react to the cytokinin substrates (i.e. IPT, CYP735A, LOG, CKX; Fig. 1), it is assumed that some components of this metabolic pathway are at least partially shared with purine metabolism, such as the purine salvage pathway (Chen, 1997; Mok and Mok, 2001; Sakakibara, 2006) and the specialized transport and distribution system would be no exception. Some members of the *Arabidopsis* purine permease (PUP) family, PUP1 and PUP2, mediate the import of adenine, caffeine, and nucleobase cytokinins, such as tZ (Gillissen *et al.*, 2000; Bürkle *et al.*, 2003).

Translocation of cytokinin nucleosides is potentially via an equilibrative nucleoside transporter (ENT). *Arabidopsis* harbours eight (*AtENT1–AtENT8*; Li *et al.*, 2003) and rice has four (*OsENT1–OsENT4*; Hirose *et al.*, 2005) ENT-homologous genes. OsENT2, which is expressed in leaf vascular bundles and phloem tissue, mediates transport of adenosine and other nucleosides, including iP riboside (iPR), and has a higher affinity for iPR than tZR (Hirose *et al.*, 2005). However, there has been no parallel

biochemical evaluation of ENT involvement in cytokinin nucleoside transport in *Arabidopsis*. Thus, the properties of AtENT-mediated cytokinin nucleoside transport were examined (Fig. 4). It had been shown that AtENT3, AtENT6, and AtENT7 mediate the transport of nucleosides such as adenosine and uridine (Wormit *et al.*, 2004). In a competition assay using the *Saccharomyces cerevisiae* expression system (Hirose *et al.*, 2005), substantial inhibition of adenosine import by iPR was observed in AtENT6-expressing yeast cells, whereas tZR was less effective (Fig. 4A). The K_m values are consistent with this assay: 17 μM for iPR (Fig. 4B) and 630 μM for tZR (Fig. 4C), which are similar to the kinetic parameters of OsENT2 (32 μM for iPR and 660 μM for tZR; Hirose *et al.*, 2005). The inhibitory effects of cytokinin nucleosides on adenosine transport were weak in AtENT3- and AtENT7-expressing yeast cells. These biochemical data suggest that AtENT6 could participate in iPR transport, and that the higher affinity of this ENT for iPR than for tZR might be involved in the spatial compartmentalization of iP and tZ-type cytokinins. A transgenic *Arabidopsis* line harbouring *AtENT6 promoter–GUS* showed that *AtENT6* is expressed in root, leaf, and flower vasculatures, and also in stomata (Fig. 4D–H), suggesting the involvement of AtENT6 in the long-distance transport of nucleosides.

Since PUP and ENT both have high affinity with adenine (AtPUP1, K_m=30 μM; Gillissen *et al.*, 2001; AtPUP2, K_m=23 μM; Bürkle *et al.*, 2003) and adenosine (AtENT6, K_m=3 μM; Wormit *et al.*, 2004), these transporters could mediate the transport of both canonical purines and nucleosides. Further studies using loss-of-function mutants should provide definitive evidence of the physiological role of cytokinin nucleoside transport candidates.



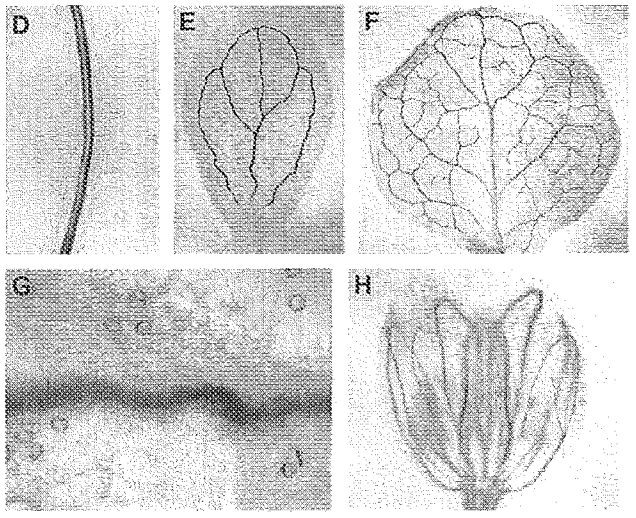


Fig. 4. Characterization of nucleoside transporters in *Arabidopsis*. (A) Inhibition of [^{3}H]adenosine uptake mediated by AtENT3, AtENT6, and AtENT7 by adenosine (+Ado, dark grey bar), iPR, (+iPR, light grey bar), tZR (+tZR, white bar), or no additive control (control, black bar). The coding regions of *AtENT* gene cDNAs were cloned into pYES2 (Invitrogen), and uptake measurements were performed as described previously (Hirose *et al.*, 2005). [^{3}H]adenosine concentration was 10 μM, and the others were 100 μM. (B, C) Determination of the kinetic parameters of AtENT6 for iPR (B) and tZR (C). [^{3}H]iPR (B) and [^{3}H]tZR (C) uptake was examined in yeast cells expressing AtENT6 by incubation for 5 min at pH 6.0. Background uptake rates (empty vector pYES2) were subtracted. Affinity constants were calculated on the basis of an Eadie–Hofstee plot (inset). Data shown are means ±SE (n=3). (D–H) Tissue specificity of *AtENT6*-promoter activity. Genomic sequences containing putative promoter regions of *AtENT6* (–1792 to +172 bp from the translational initiation codon) were cloned in-frame upstream of the GUS gene of pBI101. The GUS fusion gene was introduced into *Arabidopsis thaliana* (Col-0). Transgenic plants of the T_2 generation were grown on MGRL agar plates for approximately 21 d after germination. GUS staining was observed using an OLYMPUS BX100 microscope (OLYMPUS, Tokyo). All lines analysed showed a very similar pattern of expression, varying only in the intensity of GUS staining. Root (D), cotyledon (E), rosette leaf (F), close-up of a rosette leaf (G), and flower (H).

Concluding remarks

In this article, the focus has been on recent developments in the exploration of cytokinin metabolism, compartmentalization, and translocation. The spatial expression patterns of cytokinin metabolic genes and the biased distribution of tZ and iP-type cytokinins in vascular transporting systems strongly suggest that cytokinins act as both local and long-distance signals, and that differences in side chain structures play a specialized role, although no differences in their physiological functions have, as yet, been defined. It remains to be seen whether prosthetic group function is limited to transport and receptor, or whether there is a more subtle role for molecular structural differences. A correlation between nitrogen nutrition and cytokinins was demonstrated more than 35 years ago (Wagner and Michael, 1971), and xylem cytokinins have been thought to represent cytokinin-mediated long-distance nitrogen signalling. Previous studies demonstrated that translocation of xylem cytokinin is increased in response to nitrogen status (Rahayu *et al.*, 2005; Takei *et al.*, 2001*b*). However, a recent discussion proposed that nitrogen-dependent root cytokinin export is one mechanism involved in co-ordinating leaf growth responses to nitrogen deprivation (Dodd and Beveridge, 2006). The expression pattern of *AtIPT3*, which responds to nitrate in both root and shoot phloem, might support the idea that nitrogen-dependent synthesis of cytokinins occurs not only roots, but also in leaves. Thus, local cytokinin synthesis primarily would be important as a response to nutritional status. However, xylem-borne cytokinins must have a specialized role for regulating plant growth and development. The two pathways might co-operatively regulate plant growth and development in response to environmental changes.

Genetic tools that can be used to analyse cytokinin metabolism and translocation are becoming available, and further studies using *Arabidopsis* mutants involved in cytokinin metabolism and translocation will probably provide the clues to answer these important questions.

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